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Delayed rejection of MHC class II-disparate skin allografts in mice treated with farnesyltransferase inhibitors

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ABSTRACT

Farnesyltransferase inhibitors (FTIs), developed as anti-cancer drugs, have the potential to modulate immune responses without causing nonspecific immune suppression. We have investigated the possibility that FTIs, by affecting T cell cytokine secretion, can attenuate alloreactive immune responses. The effects of FTIs on murine alloreactive T cells were determined both *in vitro*, by measuring cytokine secretion or cell proliferation in mixed lymphocyte cultures, and *in vivo*, by performing skin allografts from *H-2*^{bm12} mice to MHC class II-disparate B6 mice. We found that two different FTIs, ABT-100 and L-744,832, blocked secretion of IFN-γ, IL-2, IL-4, and TNF-α from naïve T cells *in vitro*. ABT-100 and L-744,832 blocked cytokine production from both CD4$^+$ and CD8$^+$ naïve T cells stimulated with CD3 and CD28 antibodies, but only if the cells were pretreated with the FTIs for 48 h. Proliferation of alloreactive T cells in mixed lymphocyte cultures was blocked by either FTI. We also found that the proliferation of enriched T cells stimulated with IL-2 was blocked by ABT-100 treatment. In mice with an MHC class II-disparate skin graft, rejection of primary allografts was significantly delayed by treatment with either ABT-100 or L-744,832. Secondary rejection in mice previously primed to the alloantigen was found to be unaffected by L-744,832 treatment. We have shown that FTIs can block T cell cytokine secretion and attenuate alloreactive immune responses. The ability of FTIs to block secretion of cytokines, including IFN-γ and IL-4, from naïve T cells provides a likely biological mechanism for the specific suppression of class II MHC-mediated allorejection. These results demonstrate that FTIs may have useful immunomodulatory activity due to their ability to delay priming to alloantigens.

1. Introduction

Farnesyltransferase inhibitors (FTIs) were developed to block the function of proteins involved in oncogenic signaling by preventing their proper localization. The enzyme farnesyltransferase attaches a prenyl group to at least 17 cellular proteins, including H-Ras, K-Ras, and N-Ras, and this post-translational modification is necessary for the proper subcellular targeting and function of these substrate proteins [1]. The anti-neoplastic activity of FTIs does not depend on the presence of activated Ras oncogenic proteins and the activity of these drugs likely depends on their combined inhibitory effects on several farnesylated proteins. We have recently shown that FTIs are effective in a mouse model of mature B cell lymphoma [2], a tumor that depends on antigen receptor signaling for neoplastic growth [3]. Early clinical trials have shown that FTIs may be effective in treating hematologic malignancies and Phase III trials are ongoing for these diseases [4].

This study utilizes two structurally unrelated FTIs, L-744,832 and ABT-100. L-744,832 is a peptidomimetic inhibitor that has been shown to affect the growth and survival of tumor cells both *in vitro* [5–7] and in mouse models [8–14]. ABT-100 has been shown to have anti-neoplastic activity in xenografts of human cancer cell lines and in animal models [15–17]. This activity may be due, in part, to anti-angiogenic effects of ABT-100 [16] related to its ability to block VEGF expression [17]. Because ABT-100 and L-744,832 are not structurally related to each other, they are unlikely to share similar nonspecific effects.

Although FTIs are not known to generally suppress immune responses, several studies have suggested that they may have specific immunomodulatory activity [18–24]. FTIs do not appear to block lymphocyte activation by antigen. For example, we have found that the proliferation of murine primary B cells stimulated with anti-IgM and anti-CD40 is unaffected by concentrations of L-744,832 that block the proliferation of a B cell lymphoma [2]. The ability of HMG-CoA reductase inhibitors to block cardiac rejection and allo-antibody production in humans [25,26], first suggested that prenylated proteins may be important for allorejection (reviewed in [27]). Several farnesylated proteins are known to be involved in lymphocyte signaling, such as Ras, RhoB, and Rheb [1]. Inhibition of Ras can block IL-2 production in activated lymphocytes [28], but a Ras-independent pathway for IL-2 production has also been described [29]. A prenylation inhibitor that blocks both farnesylation and geranylgeranylation, L-778,123, can

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prevent IL-2-mediated proliferation of lymphocytes, but has no effect on IL-2 secretion [23]. FTI treatment can block secretion of inflammatory cytokines from monocytes or macrophages [18,20] as well as Th1 and Th2 cytokines from murine T cell clones [21]. The inhibition of cytokine secretion from Th1 and Th2 cell clones does not depend on Ras/MAP kinase inhibition but, rather, is due to a post-transcriptional block of cytokine synthesis [21]. In a human cancer cell line, the SCH66336 FTI has been shown to block mTOR signaling by blocking the farnesylation of Rheb [30]. It appears that ABT-100 may act through a similar mechanism to block the activation of p70S6 kinase, thereby affecting the translation of certain mRNAs, including those for certain cytokines. If FTIs can block secretion of certain cytokines under specific conditions, then this could explain their ability to affect particular immune responses without general immune suppression.

FTIs could be clinically useful as immunomodulators by affecting cytokine secretion from alloreactive T cells. The ABT-100 FTI has been shown to delay rejection of rat cardiac allografts mismatched at both class I and class II MHC [24], demonstrating that FTIs can affect alloreactive immune responses in a non-stringent rejection model. Cytokines are responsible for regulating the tolerance or rejection of allografted organs at several steps of the alloreactive immune response [31]. Manipulation of the balance of cytokines may make it possible to promote the tolerance of allografts without nonspecific immune suppression.

2. Objective

The possibility that FTIs can modulate immune responses, such as allorejection, requires further investigation. The goals of this study were to determine if FTIs could block cytokine secretion from alloreactive T cells and delay an alloreactive immune response.

3. Materials and methods

3.1. Mice and reagents

C57BL/6 mice (B6) were purchased from Charles River Laboratories. BALB/c and B6-*H2-Ab1*^{bm12} mice (bm12) were purchased from The Jackson Laboratory. All procedures involving animals were reviewed and approved by the Bucknell University IACUC. L-744,832 (BIOMOL) was dissolved at 2.5 mg/ml in DMSO and stored for no more than 1 mo at −80 °C prior to use in tissue culture experiments. For treatment of mice, L-744,832 was dissolved at 2.5 mg/ml in HBSS (Sigma), sterilized by filtration, and stored at −80 °C for no more than

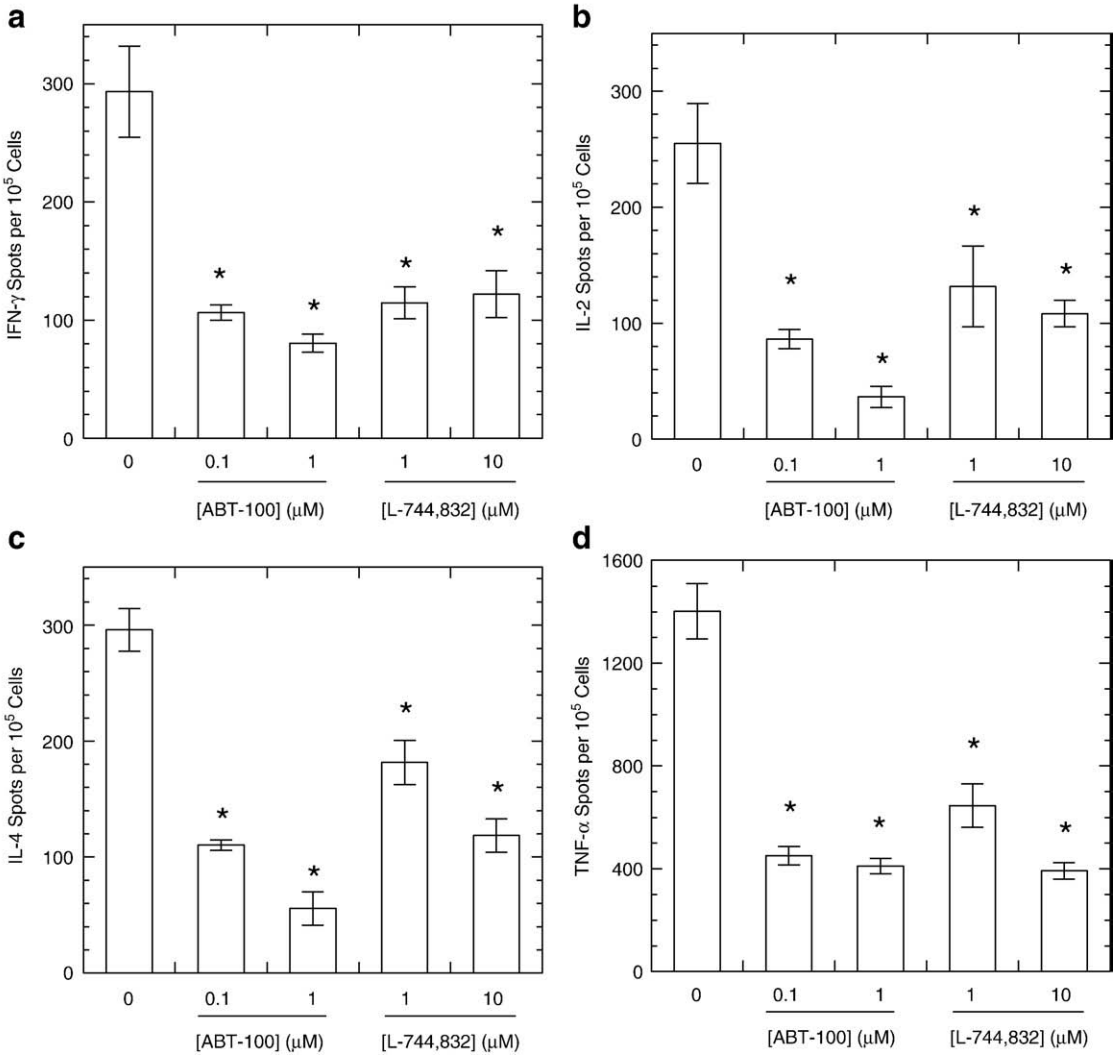

Fig. 1. Effects of FTIs on cytokine secretion from antibody-stimulated T cells. Cytokine secreting cells were detected using ELISPOT assays for IFN-γ (a), IL-2 (b), IL-4 (c), or TNF-α (d). B6 splenocytes (1×10^6 cells/ml) were treated with the indicated concentration of ABT-100 or L-744,832 beginning 24 h before stimulation. ELISPOT assays were then performed on 1×10^5 cells/well stimulated with 5 μg/ml anti-CD3 and 10 μg/ml anti-CD28 for 24 h (a) or 48 h (b–d). For each condition, the mean and standard deviation are shown for triplicate samples. An asterisk indicates a statistically significant difference between the sample and untreated cells as determined by Student's *t* test ($p<.05$).

1 wk prior to *i.v.* injection *b.i.d.* at 20 mg/kg. ABT-100, generously provided by Abbott Laboratories, was dissolved at 10 mg/ml in DMSO and stored at −20 °C prior to use in tissue culture experiments. For treatment of mice, ABT-100 was partially dissolved in ethanol at a concentration of 50 mg/ml, then diluted to 0.625 mg/ml in 0.5% hydroxypropylmethylcellulose (HPMC). The solution was adjusted to a pH of 3.5 and brought to a final concentration of 1% ethanol, 0.4% HPMC and 0.5 mg/ml ABT-100. The solution was stored at room temperature for no more than 2 weeks and provided to mice *ad libitum* as drinking water. This concentration of ABT-100 in drinking water was determined to administer an average of 100 mg/kg/day of the drug in separate experiments (data not shown).

3.2. ELISPOT assays

Splenocytes were isolated from B6 mice by macerating spleens, treating with RBC Lysis Buffer (Biolegend), and resuspending in complete RPMI media (RPMI 1640 (Mediatech) with 10% heat-inactivated FBS (PAA North America)). Cells at either 1×10^6 or 2×10^7 cells/ml were treated with ABT-100 or L-744,832 beginning 24 h prior to stimulation either with 5 μg/ml anti-CD3ε and 10 μg/ml anti-CD28 (Biolegend) or with allogeneic cells. BALB/c or bm12 stimulator cells were prepared by treating splenocytes for 20 min with 50 μg/ml mitomycin C (Sigma). Stimulator cells at 4×10^6 cells/ml were either used fresh or cryopreserved prior to use. ELISPOT kits from eBioscience were used according to the manufacturer's instructions. IFN-γ was measured for 24 h and IL-2, IL-4, and TNF-α were measured for 48 h. Data was blinded prior to manual spot counting.

3.3. Flow cytometry

Splenocytes were incubated in complete RPMI media at 1×10^6 cells/ml in the presence or absence of ABT-100 or L-744,832. Cells were stimulated by the addition of 5 μg/ml anti-CD3ε and 10 μg/ml anti-CD28. For intracellular cytokine labeling, cells were treated with 3 μg/ml brefeldin A (eBiosciences) for the final 4 h of stimulation. Cells were then blocked with anti-CD16/32 and labeled with fluorescent anti-CD4 and anti-CD8. Cells were fixed and permeabilized with Cytofix/cytoperm buffer (BD Biosciences) and then labeled with fluorescent anti-TNF-α or anti-IFN-γ antibodies (Biolegend). Flow cytometry was performed on a BD FACScan using Cellquest software for data acquisition and analysis.

3.4. Proliferation assays

Total splenocytes or splenocytes enriched for T cells by negative selection paramagnetic beads (Invitrogen) from B6 mice were suspended at 2×10^6 cells/ml and treated with ABT-100 beginning 24 h prior to stimulation. Cells were then stimulated either with 5 μg/ml anti-CD3ε and 10 μg/ml anti-CD28 or with 2 ng/ml (~10 U/ml) murine IL-2 (Peprotech). For mixed lymphocyte cultures (MLCs), B6 responder cells were stimulated with mitomycin C-treated BALB/c cells. For ^{3}H-thymidine measurements, samples were stimulated for

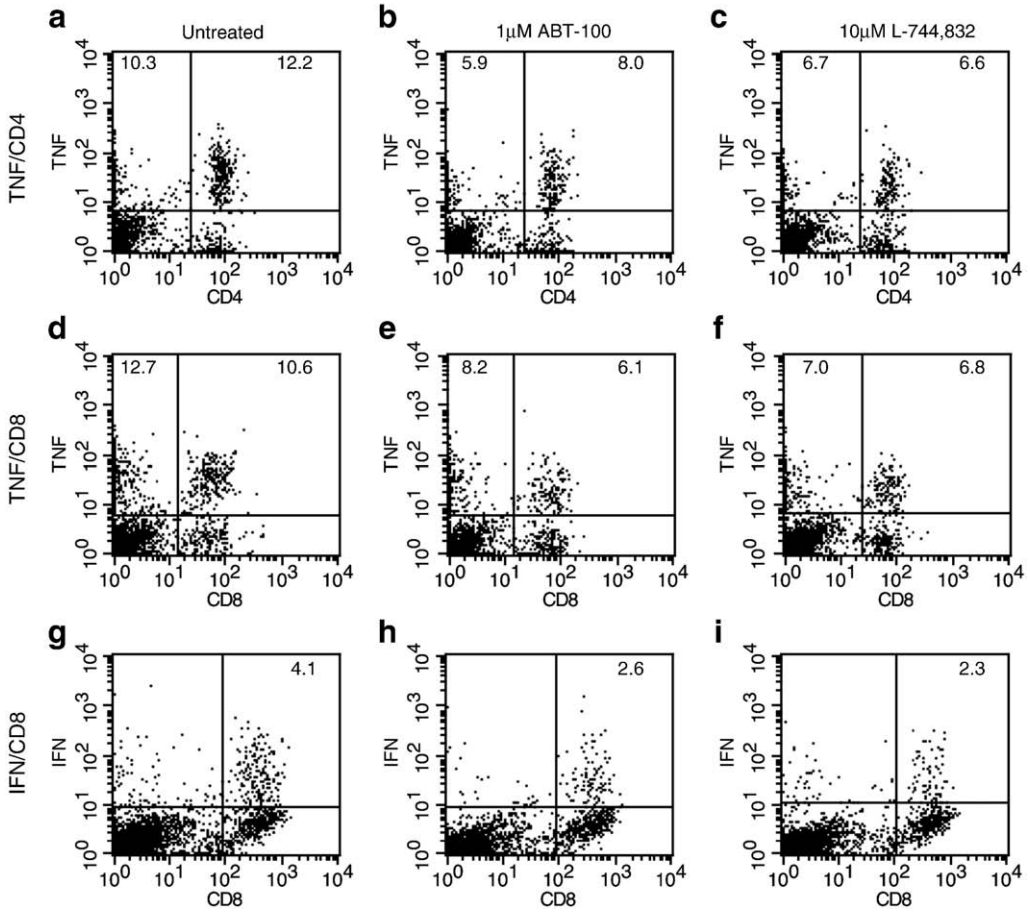

Fig. 2. Effects of FTIs on production of TNF-α and IFN-γ in $CD4^+$ and $CD8^+$ T cells. Intracellular cytokine staining was used to measure TNF-α and IFN-γ production in brefeldin A-treated cells. Splenocytes at 1×10^6 cells/ml were treated with 1.0 μM ABT-100 (b, e, and h), 10 μM L-744,832 (c, f, and i), or left untreated (a, d, and g). Cells were stimulated with 5 μg/ml anti-CD3 and 10 μg/ml anti-CD28 either 44 h later (a–f) or 24 h later (g–i). Brefeldin A was added 44 h after FTI treatment and 4 h later cells were stained with fluorescent antibodies and analyzed by flow cytometry. The percentage of viable cells in selected quadrants is shown.

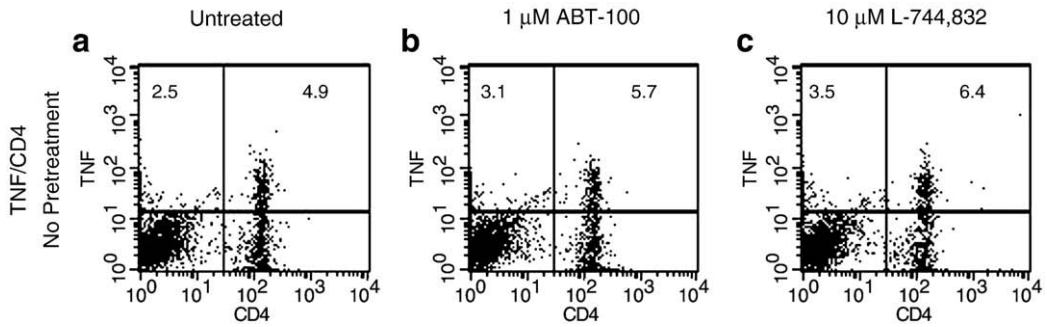

Fig. 3. Effects of FTIs on production of TNF-α in T cells without pretreatment. Intracellular cytokine staining was used to measure TNF-α production in brefeldin A-treated cells. Splenocytes at 1×10^6 cells/ml were left untreated (a), treated with 1.0 μM ABT-100 (b), or treated with 10 μM L-744,832 (c). At the same time, cells were stimulated with 5 μg/ml anti-CD3 and 10 μg/ml anti-CD28 and Brefeldin A was added. After 4 h, cells were stained with fluorescent antibodies and analyzed by flow cytometry. The percentage of viable cells in selected quadrants is shown.

72 h and then 5 μCi ^{3}H-methyl thymidine (Sigma) was added and thymidine incorporation was measured 18 h later by filter binding. For BrdU measurements, after 36 h of stimulation, BrdU was added for 24 h and incorporation was quantified using an ELISA kit (Roche Diagnostics).

3.5. Skin allografts

Skin grafts were performed using full thickness tail skin (~0.5 × 1.0 cm) from bm12 mice or B6 mice onto the dorsal trunk of B6 recipients. Seven days after surgery, bandages were removed and grafts were scored daily for rejection, which was considered complete when >90% of the donor tissue was necrotic.

4. Results

To determine whether FTI treatment could block the secretion of cytokines from naïve T cells when activated, we performed ELISPOT assays on murine splenocytes in culture. Splenocytes were harvested from B6 mice, treated with 0 to 1 μM ABT-100 or 1 to 10 μM L-744,832 and transferred to ELISPOT plates after 24 h. Antibodies to CD3 and CD28 were then used to stimulate T cells and cytokine production was measured (Fig. 1). Unstimulated cells did not secrete cytokines and CD3/CD28 stimulation promoted secretion of IFN-γ, IL-2, IL-4, and TNF-α. FTI treatment decreased the number of cytokine secreting cells for all four cytokines measured. The largest effect on cytokine secretion was seen with 1 μM ABT-100, where the treated cells were detected secreting cytokines at levels between 14% and 29% of the untreated cells. Either ABT-100 or L-744,832 treatment significantly blocked the secretion of each of these cytokines at both concentrations tested.

The results shown in Fig. 1 would be expected if the FTIs were causing cell death at the concentrations tested. To determine if these concentrations of ABT-100 or L-744,832 were cytotoxic, we measured cell death and T cell activation under the same conditions used for the ELISPOT measurements. We found that viability decreased between 5 and 15% with ABT-100 or L-744,832 treatment (unpublished results). T cell activation, as measured by CD25 expression was also not substantially affected by treatment with ABT-100 or L-744,832 (unpublished results). Together, these two results demonstrate that the concentrations of either ABT-100 or L-744,832 that were able to block the secretion of Th1 and Th2 cytokines did not block activation of T cell signaling or cause cell death.

To determine if the production of cytokines in both $CD4^+$ and $CD8^+$ T cells were affected by FTI treatment, we used intracellular cytokine staining to measure TNF-α and IFN-γ (Fig. 2). Splenocytes were treated with 1.0 μM ABT-100 or 10 μM L-744,832 or left untreated. For TNF-α measurements, after 48 h of FTI treatment, cells were stimulated with anti-CD3/anti-CD28 for 4 h. For IFN-γ measurements, after 24 h of FTI treatment, cells were stimulated with anti-CD3/anti-CD28 for 24 h. Brefeldin A was used to block cytokine secretion for the final 4 h of stimulation. Following brefeldin A treatment, cells were labeled with CD4 and CD8 fluorescent antibodies, fixed, permeabilized, and then stained with a fluorescent antibody to the appropriate cytokine. We found that TNF-α was produced by both $CD4^+$ and $CD8^+$ T cells (Fig. 2a and d) and that IFN-γ was produced predominantly by $CD8^+$ T cells (Fig. 2g). When the cells were treated with ABT-100 or L-744,832, we found that the amount of cytokine produced decreased substantially in both $CD4^+$ and $CD8^+$ T cells (Fig. 2). These results demonstrate that production of cytokines in both $CD4^+$ and $CD8^+$ naïve T cells is sensitive to FTI treatment.

We confirmed that the effects of ABT-100 or L-744,832 on cytokine secretion required preincubation of the cells with FTI. When we measured TNF-α in T cells that were stimulated immediately after FTI addition (Fig. 3), we found that neither ABT-100 nor L-744,832 blocked production of the cytokine. The inhibitory effects of FTI

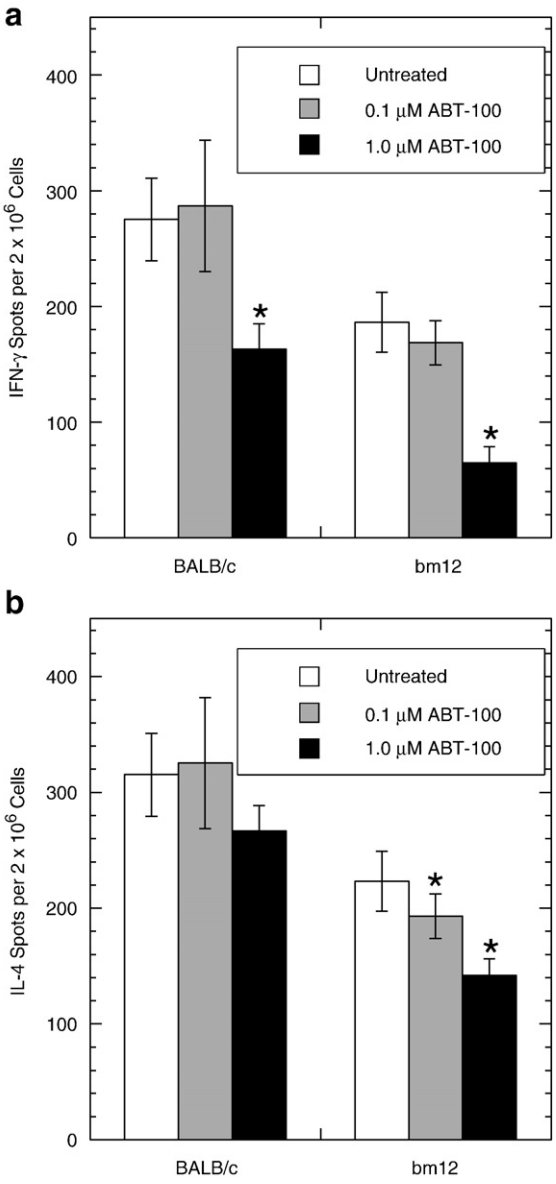

Fig. 4. Effects of FTIs on cytokine secretion from alloreactive T cells. B6 splenocytes at 2×10^7 cells/ml were treated with ABT-100 beginning 24 h before stimulation and then 2×10^6 cells/well were stimulated with 4×10^5 mitomycin C-treated BALB/c or bm12 splenocytes for 24 h (a) or 48 h (b). Cytokine secreting cells were detected using ELISPOT assays for IFN-γ (a) or IL-4 (b). For each condition, the mean and standard deviation are shown for triplicate samples. An asterisk indicates a statistically significant difference between the sample and untreated cells as determined by Student's *t* test ($p < .05$).

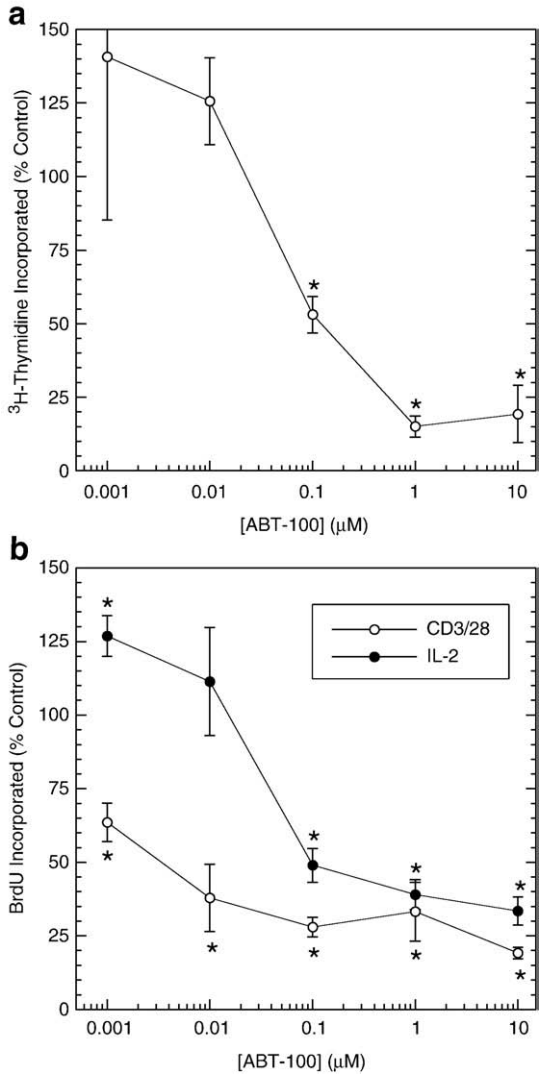

Fig. 5. Effects of ABT-100 on proliferation of T cells *in vitro*. (a) Proliferation of alloreactive T cells in MLCs was measured using ^{3}H-thymidine incorporation. B6 splenocytes at 2×10^6 cells/ml were treated with the indicated concentration of ABT-100 and then stimulated with 4×10^6 cells/ml mitomycin C-treated BALB/c stimulator cells. After 72 h of stimulation ^{3}H-thymidine incorporation was measured for 18 h by filter binding. Proliferation is shown relative to cells stimulated with allogeneic cells but not treated with ABT-100. (b) Proliferation of purified T cells stimulated with antibodies or with IL-2 was measured using BrdU incorporation. T cells were purified from B6 splenocytes by negative selection. The enriched T cells at 2×10^6 cells/ml were treated with the indicated concentration of ABT-100 for 24 h and then 2×10^5 cells/well were stimulated for 60 h with 5 μg/ml anti-CD3 and 10 μg/ml anti-CD28 or with 2 ng/ml murine IL-2. During the last 24 h of stimulation BrdU incorporation was measured with a colorimetric assay. Proliferation is shown relative to cells stimulated with antibodies or with IL-2 but not treated with ABT-100. For each condition, the mean and standard deviation are shown for triplicate samples. An asterisk indicates a statistically significant difference between the sample and untreated cells as determined by Student's *t* test ($p<.05$).

treatment required incubation of the cells for at least 48 h (unpublished results), presumably because protein turnover of farnesylated proteins is necessary.

ELISPOTs on MLCs were used to determine if the ABT-100 FTI could block cytokine secretion from naïve, alloreactive T cells. B6 splenocytes, treated with 0 to 1 μM ABT-100, were stimulated with mitomycin C-treated cells from either BALB/c mice, which are fully MHC mismatched, or bm12 mice, which have a single class II MHC mismatch (Fig. 4). We measured secretion of the Th1 cytokine, IFN-γ, after 24 h and the Th2 cytokine, IL-4, after 48 h. The allogeneic cells caused secretion of IFN-γ and IL-4 from untreated responder splenocytes. ABT-100 at 1.0 μM significantly blocked secretion of both of these cytokines from class II-disparate alloreactive T cells and secretion of IFN-γ from class I/II-disparate alloreactive T cells.

We next measured proliferation in MLCs to determine if the block in cytokine secretion by ABT-100 could affect the cytokine-dependent proliferation of alloreactive T cells. B6 splenocytes were stimulated with mitomycin C-treated BALB/c cells and proliferation was measured by ^{3}H-thymidine incorporation (Fig. 5a). Treatment of the responder cells with 0.1 μM or greater ABT-100 significantly blocked the proliferation of the alloreactive T cells stimulated by BALB/c cells. This block in proliferation was accompanied by a smaller decrease in cell viability at the highest concentration of ABT-100 tested (unpublished results), but the block in proliferation was much greater than the decrease in viability.

The decreased proliferation of FTI-treated alloreactive T cells could be caused by blocking the secretion of cytokines or by inhibiting the mitogenic effects of cytokines on lymphocytes. To determine if cytokine-stimulated cell proliferation was blocked by ABT-100 treatment, we measured T cell proliferation induced by IL-2 (Fig. 5b). B6 splenocytes were enriched for T cells by negative selection and then treated with 0 to 10 μM ABT-100. After 24 h, the T cells were then stimulated with 2 ng/ml murine IL-2 and proliferation was measured by BrdU incorporation. For comparison, we separately stimulated cells with CD3/CD28 antibodies. Both IL-2 and antibody stimulation caused proliferation of the purified T cells, and this proliferation was significantly blocked by treatment with ABT-100. Higher doses of ABT-100 were required to maximally block proliferation induced by IL-2, but both stimuli were inhibited similarly by 1 μM ABT-100 (Fig. 5b). Together with the previous data, this demonstrates that FTI treatment can both block the secretion of cytokines and block the proliferative response of T cells to mitogenic cytokines.

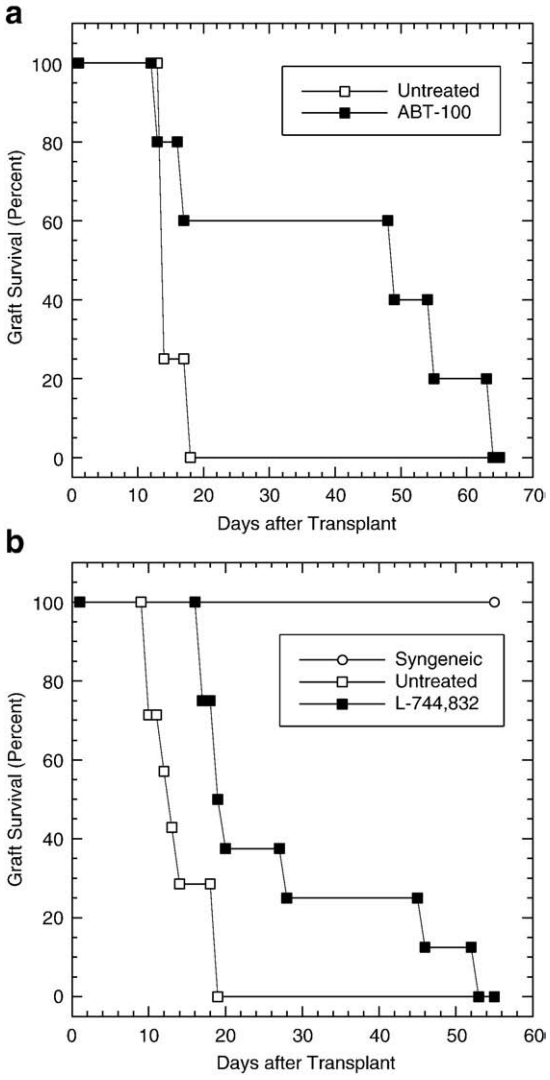

Fig. 6. Effects of FTIs on skin allograft rejection in primary responses. Skin allografts were performed using full thickness tail skin from bm12 donor mice onto the trunks of B6 mice. (a) Four transplant recipients were left untreated and 5 recipients were treated orally with 100 mg/kg/day ABT-100 beginning 2 days prior to surgery. (b) Eight transplant recipient mice were treated with 40 mg/kg/day L-744,832 *i.v.* for 7 days and 7 recipients were left untreated. Control B6 mice that received syngeneic skin grafts from B6 donor mice are shown for comparison.

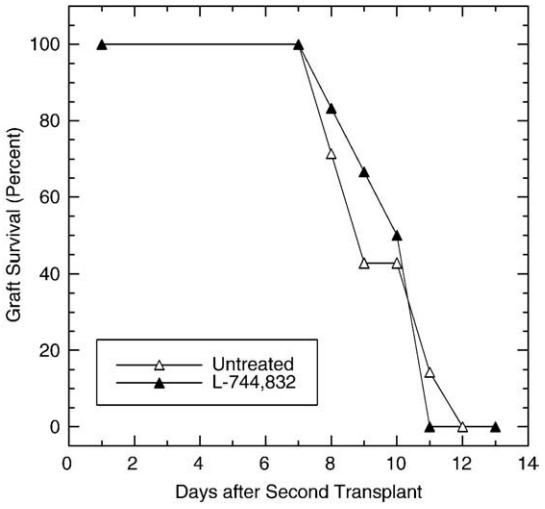

Fig. 7. Effects of L-744,832 on skin allograft rejection in a secondary alloreactive response. Skin allografts were performed on B6 mice that had previously rejected a bm12 graft approximately 4 weeks earlier. Seven of the recipient mice were left untreated and 6 of the mice were treated with 40 mg/kg/day L-744,832 *i.v.* for 7 days following the transplant.

To test if FTI treatment could affect an alloreactive immune response *in vivo*, we measured the effects of ABT-100 or L-744,832 treatment on rejection of class II MHC mismatched skin allografts in mice. Shown in Fig. 6a, 5 B6 mice were treated continuously with 0.5 mg/ml ABT-100 in their drinking water (~100 mg/kg/day) beginning 2 days before surgery. Full thickness tail skin from a bm12 mouse was engrafted onto the trunks of these mice and 4 untreated B6 mice. The mean graft survival time for the untreated mice was 13.8±1.5 days and was 38.4±23.0 days for the mice treated with ABT-100 (Fig. 6a). We also determined if the L-744,832 FTI could delay skin allograft rejection (Fig. 6b). Intravenous treatment twice daily with ~40 mg/kg/day L-744,832 began the day after surgery and continued for 7 days. The mean graft survival time for the 7 untreated mice was 12.8±3.8 days and was 26.4±14.2 days for the 8 mice treated with L-744,832. Both ABT-100 and L-744,832 treatments cause significant delays ($p<.05$) in the rejection of class II MHC mismatched skin allografts on naïve mice.

We next performed second set grafts to determine if the effects of FTI treatment on allograft rejection could also delay rejection mediated by T cells primed to the class II alloantigen (Fig. 7). Skin allograft surgeries using bm12 tail skin were performed on a cohort of B6 mice and rejection was allowed to occur without FTI treatment. Approximately 4 weeks later, a second bm12 skin allograft was performed on the opposite side of the trunk of each mouse. A group of 6 mice were treated with L-744,832 as above for 7 days beginning the day after the second surgery and 7 mice were left untreated. The mean graft survival time for the untreated mice was 9.7±1.6 days and was 10.5±1.8 days for the treated mice. The difference between these rejection times was not significant. Under the same conditions that L-744,832 delayed primary allograft rejection, this FTI did not affect allograft rejection in mice previously primed with the mismatched class II MHC alloantigen.

5. Discussion

5.1. Specificity of FTIs

By demonstrating that FTIs could affect alloreactive immune responses *in vitro* and *in vivo*, we have provided evidence that FTIs could be useful immune modulators. We used two different, structurally unrelated, FTIs for several of the experiments (Figs. 1–3 and 6) to establish that the effects we observed were specific to the inhibition of farnesyltransferase. FTIs can have off-target effects, including proteasome inhibition [32], but the observation that multiple FTIs exhibit the same effect increase the likelihood that the effects are due to the inhibition of prenylation. A previous study on cytokine secretion by murine Th1 and Th2 clones, showed that five different FTIs, including L-744,832, could block cytokine secretion [21].

The delay between FTI treatment and the effects that we have observed is consistent with the hypothesis that protein prenylation is the target of FTI treatment. Because farnesylation is an irreversible modification within the cell, protein turnover is required before the function of modified proteins is blocked. In this study, when we examined the effects of pretreating cells with FTIs for less than 24 h, we did not observe inhibition of cytokine production (Fig. 3).

At high doses, FTIs are known to inhibit geranylgeranyltransferase I [1], a prenylation enzyme closely related to farnesyltransferase. For our *in vitro* experiments, significant effects were observed at FTI concentrations that are not known to block geranylgeranyltransferase. For example, in tissue culture experiments the ABT-100 FTI has been shown to have an EC_{50} for farnesyltransferase of 0.7 nM and for geranylgeranyltransferase of >30 μM [17]. For our experiments in mice (Figs. 6 and 7), we used the maximal dose possible in order to increase the likelihood of observing an effect. For L-744,832 we used the highest dose that the mice could tolerate (unpublished results) and for ABT-100 we used the highest dose that could be reliably dissolved. Future experiments will be necessary to determine if lower doses of the drugs are effective. However, given that both FTIs showed similar efficacy and that ABT-100 has demonstrated >100,000-fold selectivity for farnesyltransferase inhibition over geranylgeranyltransferase [17], it is most likely that the effects we have observed are due to farnesyltransferase inhibition.

The farnesylated protein or proteins that are the functional target of FTI treatment are not known. Up to 17 proteins are farnesylated within the cell and several of them are known to be involved in lymphocyte signaling [1]. Interestingly, transgenic mice that express dominant negative H-Ras show delayed rejection of skin allografts that are fully mismatched at MHC class I and II [33]. This observation suggests that H-Ras inhibition may be involved in the action of FTIs during allograft rejection. However, the inhibition of cytokine secretion by FTI treatment does not appear to involve Ras inhibition [21]. Therefore, it appears that some combination of farnesylated proteins are the likely targets of FTI inhibition, as has been shown for the anti-neoplastic activity of these drugs [1].

5.2. FTIs block cytokine production and cytokine-induced proliferation in T cells

The ability of ABT-100 and L-744,832 to block secretion of cytokines provides a possible mechanism for their specific immunosuppression activity. ABT-100 prevented secretion of IFN-γ, IL-2, IL-4, and TNF-α from naïve T cells, extending the previous observations that this FTI blocks secretion of IFN-γ and IL-2 from murine Th1 clones and secretion of IL-4 and IL-5 from murine Th2 clones [21]. We confirmed that FTIs were blocking cytokine production, and not simply killing the cytokine-producing cells, by measuring cell viability and by directly measuring cytokine synthesis within viable T cells (Fig. 2). In addition to affecting $CD4^+$ T cells, we have demonstrated that ABT-100 can block secretion of TNF-α and IFN-γ from naïve $CD8^+$ T cells (Figs. 1 and 2). This result is consistent with other observations that TNF-α secretion can be blocked by FTIs both *in vitro* and *in vivo* [18,20]. A less robust, but still significant, inhibition of cytokine secretion was seen when allogeneic stimulator cells were used instead of anti-CD3 and anti-CD28 antibodies (Fig. 4). Our further observation that the mitogenic response of T cells to IL-2 is blocked by ABT-100 (Fig. 5b) indicates that the effects of cytokines on target cells may also be blocked by FTIs. The block in proliferation is not mediated by preventing the expression of high affinity IL-2 receptors on activated T cells, which we have found is unaffected by FTI treatment (unpublished results). This observation is consistent with previous results showing that ABT-100 does not effect expression of CD25 or other activation markers on phytohemagglutinin-activated human peripheral blood mononuclear cells [24]. Together, our results show that FTIs may have several effects on immune responses by modulating the production of cytokines from a variety of cell types and the mitogenic responses of cells to cytokines.

5.3. FTIs block proliferation of alloreactive T cells in MLCs

FTI treatment was found to block alloreactive cell proliferation in both $CD4^+$ and $CD8^+$ T cells. Using BALB/c stimulator cells mismatched at both class I and II MHC, ABT-100 was found to inhibit proliferation by up to 85% (Fig. 5a). This provides clear evidence that the effects of FTI treatment on cytokine secretion can delay an alloreactive immune response and that the target of FTI action is the alloreactive T cell. While the *in vitro* results do not preclude additional effects on the recognition or effector phases of allorejection, a principal target of FTI action appears to be the expansion phase. Proliferation of T cells under these conditions is dependent on the mitogenic activity of cytokines; we also found that T cell proliferation induced by IL-2 was blocked by ABT-100 (Fig. 5b). We conclude that FTIs can affect both the secretion of cytokines and their mitogenic stimulation of lymphocytes.

5.4. FTI treatment can delay alloreactive rejection in vivo

Treatment with either ABT-100 or L-744,832 delayed rejection of class II MHC-disparate primary allografts (Fig. 6), directly demonstrating that FTIs can affect immune responses *in vivo*. Our results indicate that FTIs may delay the expansion of $CD4^+$ T cells once primed to alloantigens by affecting cytokine secretion. By delaying the expansion of the alloreactive T cells (Fig. 5), FTIs can slow the rejection of primary allografts but do not affect secondary rejection (Fig. 7), because the expansion of alloreactive clones has already occurred.

FTIs may also affect priming of the alloreactive immune response. Priming of $CD4^+$ T cells to MHC class II alloantigens involves several steps, including the direct pathway of antigen presentation, recognition of MHC by $CD4^+$ T cells, and activation of alloreactive T cells. Our results from *in vitro* assays indicate that FTIs can block the cytokine secretion that is involved in both the proliferation and differentiation of alloreactive T cells after they have been primed. While we cannot exclude possible effects of FTIs on antigen presentation or recognition of alloantigens, the effects of FTIs on cytokine secretion are sufficient to explain the delay in allograft rejection.

5.5. Possible mechanisms of FTI action in allograft rejection

We chose a stringent model of MHC class II-mediated skin allograft rejection because it had previously been shown to be dependent on the secretion of a single cytokine, IFN-γ. B6 mice deficient in IFN-γ or treated with anti-IFN-γ antibodies are not capable of rejecting bm12 allografts, although they exhibit normal allorejection of class I mismatched and fully mismatched grafts [34,35]. We demonstrated that treatment with either ABT-100 or L-744,832 can delay bm12 graft rejection in mice (Fig. 5). Therefore, we can conclude that FTI treatment affects MHC II allorecognition and the activation of naïve $CD4^+$ T cells. We provide two possible mechanisms for this activity of xFTIs involving IFN-γ. By blocking the secretion of IFN-γ from activated naïve $CD4^+$ T cells, FTIs may prevent the generation of a proinflammatory environment and the IFN-γ-dependent secretion of the Mig chemokine, which is also required for bm12 graft rejection in B6 mice [36]. Second, FTIs may block the effects of IFN-γ and other cytokines on the proliferation and/or survival of effector cells, as we found for IL-2 (Fig. 5b).

It is likely that FTI treatment can affect other cytokines in addition to those measured in this study. We demonstrated that FTI treatment affected the Th2 cytokine, IL-4 (Fig. 1). Class II mismatched graft rejection involves secretion of both IL-4 and IL-5 by Th2 cells [37,38]. A third possible mechanism of action of FTIs on graft rejection involves these Th2 cytokines. FTI treatment has the potential to affect the initial coordination of the alloreactive response by stimulated naïve T cells and alter their differentiation into Th1, Th2, and Treg cells. Inhibition of Ras has been shown to increase FoxP3 expression in a human T cell line [39], suggesting that FTI treatment could increase the number of Treg cells induced during an immune response, presumably at the expense of Th1 or Th2 cells. To determine if FTI treatment affects the balance of Th1, Th2, and Treg cells generated, future studies will be necessary to measure the effects of FTIs on cytokine production *in vivo* and the generation of allospecific effector cells in graft-draining lymph nodes.

Although several mechanisms may be involved in delaying allograft rejection, our results suggest that the block in IFN-γ secretion may be the most critical. The observation that second set rejection was not affected by L-744,832 treatment (Fig. 7) demonstrated that, after $CD4^+$ T cells had been primed to the MHC II alloantigen, the FTI no longer significantly delayed the rejection process. This observation is similar to that seen in IFN-γ knockout mice that readily reject bm12 skin allografts only after priming to the alloantigen [34]. The ability of FTI-treated mice to rapidly reject second set grafts demonstrates that FTI treatment does not block the effector mechanisms responsible for tissue cytotoxicity, including lymphocyte migration and the production of proinflammatory cytokines within the skin graft. Our results suggest that once an alloreactive response had taken place during the primary rejection, treatment with FTIs was unable to delay the strong allorejection response of the primed effector T cells. This observation may suggest that FTIs would be less useful to treat ongoing alloreactive immune responses than as preventative therapy.

5.6. Implications

Our results have particular implications for the treatment of malignancies by hematopoietic stem cell (HSC) transplantation and the induction of tolerance following solid organ transplantation. By including an FTI in the preparative or maintenance phases of HSC therapy, we suggest that it might be possible to utilize both the direct anti-neoplastic and the potential graft versus host disease inhibitory activities of an FTI to improve outcomes. Through similar immunomodulatory mechanisms, it may be possible to use FTIs to delay allorejection and induce tolerance following solid organ transplantation.

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References

[1] Basso AD, Kirschmeier P, Bishop WR. Lipid posttranslational modifications. Farnesyl transferase inhibitors. J Lipid Res 2006;47(1):15–31.
[2] Field KA, Charoenthongtrakul S, Bishop JM, Refaeli Y. Farnesyl transferase inhibitors induce extended remissions in transgenic mice with mature B cell lymphomas. Mol Cancer 2008;7(1):39.
[3] Refaeli Y, Young RM, Turner BC, Duda J, Field KA, Bishop JM. The B-cell antigen receptor and overexpression of MYC can cooperate in the genesis of B-cell lymphomas. PLoS Biology 2008;6(6):e152.
[4] Harousseau JL. Farnesyltransferase inhibitors in hematologic malignancies. Blood Rev 2007;21(4):173–82.
[5] Sepp-Lorenzino L, Ma Z, Rands E, Kohl NE, Gibbs JB, Oliff A, et al. A peptidomimetic inhibitor of farnesyl:protein transferase blocks the anchorage-dependent and -independent growth of human tumor cell lines. Cancer Res 1995;55(22):5302–9.
[6] Feldkamp MM, Lau N, Guha A. Growth inhibition of astrocytoma cells by farnesyl transferase inhibitors is mediated by a combination of anti-proliferative, pro-apoptotic and anti-angiogenic effects. Oncogene 1999;18(52):7514–26.
[7] van Golen KL, Bao L, DiVito MM, Wu Z, Prendergast GC, Merajver SD. Reversion of RhoC GTPase-induced inflammatory breast cancer phenotype by treatment with a farnesyl transferase inhibitor. Mol Cancer Ther 2002;1(8):575–83.
[8] Sirotnak FM, Sepp-Lorenzino L, Kohl NE, Rosen N, Scher HI. A peptidomimetic inhibitor of Ras functionality markedly suppresses growth of human prostate tumor xenografts in mice. Prospects for long-term clinical utility. Cancer Chemother Pharmacol 2000;46(1):79–83.
[9] Kohl NE, Omer CA, Conner MW, Anthony NJ, Davide JP, deSolms SJ, et al. Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in Ras transgenic mice. Nat Med 1995;1(8):792–7.

[10] Barrington RE, Subler MA, Rands E, Omer CA, Miller PJ, Hundley JE, et al. A farnesyltransferase inhibitor induces tumor regression in transgenic mice harboring multiple oncogenic mutations by mediating alterations in both cell cycle control and apoptosis. Mol Cell Biol 1998;18(1):85–92.
[11] Mangues R, Corral T, Kohl NE, Symmans WF, Lu S, Malumbres M, et al. Antitumor effect of a farnesyl protein transferase inhibitor in mammary and lymphoid tumors overexpressing N-ras in transgenic mice. Cancer Res 1998;58(6):1253–9.
[12] Mahgoub N, Taylor BR, Gratiot M, Kohl NE, Gibbs JB, Jacks T, et al. *In vitro* and *in vivo* effects of a farnesyltransferase inhibitor on Nf1-deficient hematopoietic cells. Blood 1999;94(7):2469–76.
[13] Norgaard P, Law B, Joseph H, Page DL, Shyr Y, Mays D, et al. Treatment with farnesyl-protein transferase inhibitor induces regression of mammary tumors in transforming growth factor (TGF) alpha and TGF alpha/neu transgenic mice by inhibition of mitogenic activity and induction of apoptosis. Clin Cancer Res 1999;5 (1):35–42.
[14] Omer CA, Chen Z, Diehl RE, Conner MW, Chen HY, Trumbauer ME, et al. Mouse mammary tumor virus-Ki-rasB transgenic mice develop mammary carcinomas that can be growth-inhibited by a farnesyl:protein transferase inhibitor. Cancer Res 2000;60(10):2680–8.
[15] Carloni V, Vizzutti F, Pantaleo P. Farnesyltransferase inhibitor, ABT-100, is a potent liver cancer chemopreventive agent. Clin Cancer Res 2005;11(11):4266–74.
[16] Ferguson D, Rodriguez LE, Palma JP, Refici M, Jarvis K, O'Connor J, et al. Antitumor activity of orally bioavailable farnesyltransferase inhibitor, ABT-100, is mediated by antiproliferative, proapoptotic, and antiangiogenic effects in xenograft models. Clin Cancer Res 2005;11(8):3045–54.
[17] Gu WZ, Joseph I, Wang YC, Frost D, Sullivan GM, Wang L, et al. A highly potent and selective farnesyltransferase inhibitor ABT-100 in preclinical studies. Anticancer Drugs 2005;16(10):1059–69.
[18] Xue X, Lai KT, Huang JF, Gu Y, Karlsson L, Fourie A. Anti-inflammatory activity *in vitro* and *in vivo* of the protein farnesyltransferase inhibitor tipifarnib. J Pharmacol Exp Ther 2006;317(1):53–60.
[19] Takada Y, Khuri FR, Aggarwal BB. Protein farnesyltransferase inhibitor (SCH 66336) abolishes NF-kappaB activation induced by various carcinogens and inflammatory stimuli leading to suppression of NF-kappaB-regulated gene expression and up-regulation of apoptosis. J Biol Chem 2004;279(25):26287–99.
[20] Na HJ, Lee SJ, Kang YC, Cho YL, Nam WD, Kim PK, et al. Inhibition of farnesyltransferase prevents collagen-induced arthritis by down-regulation of inflammatory gene expression through suppression of p21(Ras)-dependent NF-kappaB activation. J Immunol 2004;173(2):1276–83.
[21] Marks RE, Ho AW, Robbel C, Kuna T, Berk S, Gajewski TF. Farnesyltransferase inhibitors inhibit T-cell cytokine production at the posttranscriptional level. Blood 2007;110(6):1982–8.
[22] Koh WS, Yoon SY, Kwon BM, Jeong TC, Nam KS, Han MY. Cinnamaldehyde inhibits lymphocyte proliferation and modulates T-cell differentiation. Int J Immunopharmacol 1998;20(11):643–60.
[23] Si MS, Ji P, Tromberg BJ, Lee M, Kwok J, Ng SC, et al. Farnesyltransferase inhibition: a novel method of immunomodulation. Int Immunopharmacol 2003;3(4):475–83.
[24] Si MS, Ji P, Lee M, Kwok J, Kusumoto J, Naasz E, et al. Potent farnesyltransferase inhibitor ABT-100 abrogates acute allograft rejection. J Heart Lung Transplant 2005;24(9):1403–9.
[25] Kobashigawa JA, Katznelson S, Laks H, Johnson JA, Yeatman L, Wang XM, et al. Effect of pravastatin on outcomes after cardiac transplantation. N Engl J Med 1995;333(10):621–7.
[26] Maggard MA, Johnson C, Wang T, Terasaki P, Kupiec-Weglinski JW, Busuttil RW, et al. Inhibition of transplant vasculopathy is associated with attenuated host allo-antibody response. Transplant Proc 1999;31(1–2):728–9.
[27] Greenwood J, Steinman L, Zamvil SS. Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. Nat Rev Immunol 2006;6(5):358–70.
[28] Rayter SI, Woodrow M, Lucas SC, Cantrell DA, Downward J. p21ras mediates control of IL-2 gene promoter function in T cell activation. EMBO J 1992;11(12):4549–56.
[29] Lafont V, Ottones F, Liautard J, Favero J. Evidence for a p21(Ras)/Raf-1/MEK-1/ERK-2-independent pathway in stimulation of IL-2 gene transcription in human primary T lymphocytes. J Biol Chem 1999;274(36):25743–8.
[30] Basso AD, Mirza A, Liu G, Long BJ, Bishop WR, Kirschmeier P. The farnesyl transferase inhibitor (FTI) SCH66336 (lonafarnib) inhibits Rheb farnesylation and mTOR signaling. Role in FTI enhancement of taxane and tamoxifen anti-tumor activity. J Biol Chem 2005;280(35):31101–8.
[31] Walsh PT, Strom TB, Turka LA. Routes to transplant tolerance versus rejection; the role of cytokines. Immunity 2004;20(2):121–31.
[32] Efuet ET, Keyomarsi K. Farnesyl and geranylgeranyl transferase inhibitors induce G1 arrest by targeting the proteasome. Cancer Res 2006;66(2):1040–51.
[33] Funeshima-Fuji N, Fujino M, Kimura H, Takahara S, Nakayama T, Ezaki T, et al. Survival of skin allografts is prolonged in mice with a dominant-negative H-Ras. Transpl Immunol 2008;18(4):302–6.
[34] Ring GH, Saleem S, Dai Z, Hassan AT, Konieczny BT, Baddoura FK, et al. Interferon-gamma is necessary for initiating the acute rejection of major histocompatibility complex class II-disparate skin allografts. Transplantation 1999;67(10):1362–5.
[35] Rosenberg AS, Finbloom DS, Maniero TG, Van der Meide PH, Singer A. Specific prolongation of MHC class II disparate skin allografts by *in vivo* administration of anti-IFN-gamma monoclonal antibody. J Immunol 1990;144(12):4648–50.
[36] Koga S, Auerbach MB, Engeman TM, Novick AC, Toma H, Fairchild RL. T cell infiltration into class II MHC-disparate allografts and acute rejection is dependent on the IFN-gamma-induced chemokine Mig. J Immunol 1999;163(9):4878–85.
[37] Le Moine A, Surquin M, Demoor FX, Noel JC, Nahori MA, Pretolani M, et al. IL-5 mediates eosinophilic rejection of MHC class II-disparate skin allografts in mice. J Immunol 1999;163(7):3778–84.
[38] Surquin M, Le Moine A, Flamand V, Rombaut K, Demoor FX, Salmon I, et al. IL-4 deficiency prevents eosinophilic rejection and uncovers a role for neutrophils in the rejection of MHC class II disparate skin grafts. Transplantation 2005;80 (10):1485–92.
[39] Mor A, Keren G, Kloog Y, George J. N-Ras or K-Ras inhibition increases the number and enhances the function of Foxp3 regulatory T cells. Eur J Immunol 2008;38(6):1493–502.